PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/18, C11B 3/00 — A1

(11) International Publication Number: **WO 98/18912**

(43) International Publication Date: 7 May 1998 (07.05.98)

(21) International Application Number: PCT/DK97/00490

(22) International Filing Date: 30 October 1997 (30.10.97)

(30) Priority Data:
1215/96 31 October 1996 (31.10.96) DK

(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK–2880 Bagsværd (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: HASIDA, Miyoko [JP/JP]; 5–3–311, Hinode, Urayasu–shi, Chiba–ken 279 (JP). TSUTSUMI, Noriko [JP/JP]; 3–2–16, Higashisagano, Ichikawa–shi, Chiba–ken 272 (JP). HALKIER, Torben [DK/DK]; Hestkøbvej 11E, DK–3460 Birkerød (DK). STRINGER, Mary, Ann [US/DK]; Rosenvængets Hovedvej 42, 1.tv., DK–2100 København Ø (DK).

(74) Common Representative: NOVO NORDISK A/S; Att: Corporate Patents, Novo Allé, DK–2880 Bagsværd (DK).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NOVEL PHOSPHOLIPASE, PRODUCTION AND USE THEREOF

(57) Abstract

An acidic phospholipase is obtained from a strain of the genus *Hyphozyma*. It is able to hydrolyze both fatty acyl groups in intact phospholipid. Advantageously, it has no lipase activity and is active at very low pH; these properties make it very suitable for use in oil degumming, as enzymatic and alkaline hydrolysis (saponification) of the oil can both be suppressed. The phospholipase is not membrane bound, making it suitable for commercial production and purification.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

NOVEL PHOSPHOLIPASE, PRODUCTION AND USE THEREOF

TECHNICAL FIELD

This invention relates to a novel phospholipase, DNA encoding it and to its production and use.

BACKGROUND ART

Phospholipids, such as lecithin or phosphatidylcholine, consist of glycerol esterified with two fatty acids in an outer (sn-1) and the middle (sn-2) positions and esterified with phosphoric acid in the third position; the phosphoric acid, in turn, may be esterified to an amino-alcohol. Phospholipases are enzymes which participate in the hydrolysis of phospholipids. Several types of phospholipase activity can be distinguished, including phospholipase A1 and A2 which hydrolyze one fatty acyl group (in the sn-1 and sn-2 position, respectively) to form lysophospholipid; and lysophospholipase (or phospholipase B) which can hydrolyze the remaining fatty acyl group in lysophospholipid. This invention relates to a phospholipase that has the ability to hydrolyze both fatty acyl groups in a phospholipid. Enzymes with this activity are also some times called phospholipase B.

Enzymes with phospholipase B activity have been reported from various fungal sources, including *Penicillium notatum* (also known as *P. chrysogenum*; N. Kawasaki, J. Biochem., 77, 1233-44, 1975; N. Masuda et al., Eur. J. Biochem., 202, 783-787, 1991), *Saccharomyces cerevisiae* (M. Ichimasa et al., Agric. Biol. Chem., 49 (4), 1083-89, 1985; F. Paultauf et al., J. Biol. Chem., 269, 19725-30, 1994), *Torulaspora delbrueckii* (old name *Saccharomyces rosei*; Y. Kuwabara, Agric. Biol. Chem., 52 (10), 2451-58, 1988; FEMS, Microbiol. Letters, 124, 29-34), *Schizosaccharomyces pombe* (H. Oishi et al., Biosci. Biotech. Biochem., 60 (7), 1087-92, 1996), *Aspergillus niger* (Technical Bulletin, G-zyme™ G999, Enzyme Bio-Systems Ltd.) and *Corticium centrifugum* (S. Uehara et al., Agric. Biol. Chem., 43 (3), 517-525, 1979).

It is known to use phospholipase in, e.g., enzymatic oil degumming (US 5,264,367, Metallgesellschaft, Röhm), treatment of starch hydrolysate (particularly from wheat starch) to improve the filterability (EP 219,269, CPC International) and as an additive to bread dough to improve the elasticity of the bread (US 4,567,046, Kyowa Hakko).

It is the object of this invention to provide an improved phospholipase for use in such processes.

STATEMENT OF THE INVENTION

The present inventors have found that an acidic phospholipase can be obtained from a strain of the genus *Hyphozyma*. It is able to hydrolyze both fatty acyl groups in intact phospholipid. Advantageously, it has no lipase activity and is active at very low pH; these properties make it very suitable for use in oil degumming, as enzymatic and alkaline hydrolysis (saponification) of the oil can both be suppressed. The phospholipase is not membrane bound, making it suitable for commercial production and purification.

WO 93/24619 (Novo Nordisk) discloses a lipase from *Hyphozyma sp.* LF-132 (CBS 648.91), but the production of phospholipase by this genus has never been reported. We have found that the phospholipase of this invention can be obtained from the same strain as the known lipase, and that the two enzymes can be separated.

Accordingly, a first aspect of the invention provides an isolated phospholipase which is able to hydrolyze both fatty acyl groups in a phospholipid, is derivable from a strain of *Hyphozyma*, and has optimum phospholipase activity at about 50°C and pH 3 measured at the conditions described in Example 3.

The invention also provides an isolated phospholipase which is able to hydrolyze both fatty acyl groups in a phospholipid, and is a polypeptide comprising at its N-terminal a partial amino acid sequence which is the sequence shown in positions 1-497 of SEQ ID NO: 11, or is at least 50 % identical therewith

In another aspect, the invention provides an isolated phospholipase which is able to hydrolyze both fatty acyl groups in a phospholipid, and is a polypeptide containing amino acid sequences which are at least 50 % identical with the amino acid sequences shown in SEQ ID NO: 1-8, disregarding Xaa.

The invention further provides an isolated DNA sequence which encodes said phospholipase.

Yet another aspect of the invention provides a method of producing a phospholipase, comprising cultivation of a phospholipase-producing strain of *Hyphozyma* in a suitable nutrient medium, followed by recovery of the phospholipase

A further aspect of the invention provides a method for producing a phospholipase, comprising isolating a DNA sequence encoding the phospholipase from a phospholipase-producing strain of *Hyphozyma*, combining the DNA fragment with appropriate expression signal(s) in an appropriate vector, transforming a suitable heterologous host organism with the vector, cultivating the transformed host organism under conditions leading to expression of the phospholipase, and recovering the phospholipase from the culture medium

The invention also provides use of said phospholipase in a process comprising treatment of a phospholipid or lysophospholipid with the phospholipase so as to hydrolyze fatty acyl groups.

Finally, the invention provides a process for reducing the content of phospholipid in a vegetable oil, comprising treating the oil with an aqueous dispersion of an acidic phospholipase at pH 1.5-3 so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil.

BRIEF DESCRIPTION OF DRAWINGS

Figs. 1, 2 and 3 show the temperature profile, pH profile and thermostability, respectively, of phospholipase from *Hyphozyma sp.* CBS 648.91. Further details are given in Example 3.

Fig. 4a-d gives a comparison of SEQ ID NO: 11 with 3 prior-art sequences.

DETAILED DISCLOSURE OF THE INVENTION

Phospholipase

The phospholipase of the invention is able to hydrolyze both acyl groups in a phospholipid molecule (such as phosphatidyl choline or lecithin) without intermediate accumulation of lysophospholipid and is also able to hydrolyze the fatty acyl group of a lysophospholipid (such as lysophosphatidyl choline or lyso-lecithin). Advantageously, the phospholipase of the invention is not membrane bound.

A preferred enzyme is derived from *Hyphozyma sp.* strain CBS 648.91. Its molecular weight is about 94 kDa by SDS, about 87 kDa by gel filtration, and 92 kDa by mass spectrometry. It is believed to be glycosylated. It has an iso-electric point of about 5.6. It has no lipase activity, i.e. it does not hydrolyze triglycerides.

The influence of pH and temperature on the activity of this phospholipase is shown in Fig. 1 and 2. As shown in these figures, the enzyme has optimum activity at about pH 3 and 50°C.

Fig. 3 shows the thermostability of this enzyme, expressed as the residual activity after 10 minutes at pH 7 at various temperatures. It is seen that the enzyme retains more than 90 % activity at temperatures up to 50°C, more than 75% up to 60°C and more than 50% up to 70°C.

Phospholipase Activity Assay

Two different units are used in this specification:

1 unit (phospholipase activity unit) is the amount of phospholipase that releases one μ(micro)-mole of fatty acid per minute from DPPC (dipalmitoyl phosphatidylcholine) at 40°C and pH 4. The amount of released fatty acid is determined by NEFA-C test Wako.

1 International Unit (IU) is the amount of phospholipase that releases one μ(micro)-equivalent of free fatty acid per minute from egg yolk in the presence of calcium and deoxycholate at pH 8.0 and 40°C in a pH-stat. The released fatty acids are titrated with 0.1 N sodium hydroxide and the base volume is monitored as a function of time.

Assay for action pattern of phospholipase

The following test is used to identify if a given enzyme has the ability to hydrolyze both fatty acyl groups of a phospholipid without the accumulation of lysophospholipid.

A substrate solution is prepared containing 2% L-α(alpha)-phosphatidylcholine, dipalmitoyl (product of Wako Pure Chemical Industries Ltd.) and 2% Triton X-100. A buffer solution is prepared containing 0.4 M citrate buffer (pH 5). Enzyme solutions are prepared containing various amounts of the sample to be analyzed.

0.5 ml of the substrate solution, 0.25 ml of the buffer solution and 0.05 ml of 0.1 N $CaCl_2$ are mixed and incubated at 40°C. 0.1 ml of the enzyme solution is added and incubated for 1 hour. The reaction is terminated by adding 0.1 ml of 1 N HCl.

2 ml of $CHCl_3$-methanol (1:1) is added to the reaction mixture and mixed vigorously. Approx. 1 μ(micro)l of the $CHCl_3$-methanol is taken and applied to a TLC rod (in triplicate or quadruplicate). the TLC rods are dried and developed for 45 minutes with $CHCl_3$: methanol : NH_3 (25% solution) = 65:25:5. After the development, the rods are scanned by TLC-FID (Iatroscan), and the chromatograms are integrated.

The amounts of palmitate, the substrate, lysophosphatidyl choline (LPC) and glycerophosphatidyl choline (GPC) are calculated from the areas of peaks appearing in that order.

The result of the test is considered positive if GPC is formed without any LPC formation.

Amino acid sequence

Partial sequences SEQ ID NO: 1-8 were determined by sequencing of phospholipase from *Hyphozyma sp.* CBS 648.91 after enzymatic hydrolysis. In these sequences, Xaa represents an amino acid that could not be determined. SEQ ID NO:

1 is an N-terminal sequence, and the others are internal sequences. Xaa in SEQ ID NO: 1 is believed to be a Pro residue. Xaa in SEQ ID NO: 3, 7 and 8 and both Xaa in SEQ ID NO: 5 are believed to be glycosylated Asn residues.

A nearly complete DNA sequence (SEQ ID NO: 9) was determined for the gene encoding the phospholipase from *Hyphozyma sp.* CBS 648.91. This sequence was determined from the genomic locus and includes an open reading frame of 552 amino acids and 213 base pairs of sequence upstream of the putative translation initiation codon. The methods used for sequence isolation and determination are well known in the art. Details are given in the examples.

The long, uninterrupted open reading frame identified in this sequence was translated and compared to the partial peptide sequences SEQ ID NO: 1-8. The translated sequence was identical to seven of the partial peptide sequences at all positions, SEQ ID NO:1-7, and overlapped the most distal partial peptide sequence, SEQ ID NO: 8 by 10 amino acids. By combining the translation with partial peptide NO: 8, a sequence of 573 amino acid residues (shown as SEQ ID NO: 11) has been determined. The amino terminus of the mature peptide is determined by comparison with SEQ ID NO: 1. The sequenced open reading frame extends upstream an additional 115 amino acids. There is only one Met codon in this region, 76 amino acids from the start of the mature peptide (position -76). The 14 amino acids immediately following this methionine residue appear to constitute a secretion signal sequence (G. von Heijne, Nucleic Acids Res, 14, 4683-4690, 1986), indicating both that this is the translation initiation codon and that the encoded protein is secreted. The intervening 61 amino acids must constitute a propeptide.

The peptide sequence from Hyphozyma was aligned with the phospholipase B sequences from three other fungi, Penicillium notatum (Genbank X60348), Saccharomyces cerevisiae (Genbank L23089) and Torulaspora delbrueckii (Genbank D32134), as shown in Fig. 4a-d. In this alignment a dash (-) indicates an inserted gap, a circle (o) above the alignment marks a position at which the same amino acid is found in all proteins, and a vertical line (|) above the alignment indicates similar residues in all proteins. The portion of the Hyphozyma phospholipase sequence we have determined is 38% identical to the phospholipase from Penicillium notatum, 37% identical to the phospholipase from Saccharomyces cerevisiae, and 38% identical to the phospholipase from Torulaspora delbrueckii. The full length Penicillium, Saccharomyces, and Torulaspora sequences extend from 112 to 145 residues further than the partial Hyphozyma sequence, suggesting that the full length for the translated Hyphozyma peptide is approximately 700 amino acid residues.

Thus, the phospholipase of the invention may contain an N-terminal sequence as shown at positions 1-497 of SEQ ID NO: 11 or a sequence derived therefrom by substitution, deletion or insertion of one or more amino acids. The derived sequence may be at least 50 % identical, e.g. at least 60%, preferably at least 70%, especially at least 80 or at least 90% identical with said partial sequence. The phospholipase of the invention may contain a further 150-250 (e.g. 180-220) amino acid residues at the C-terminal

Microorganism

The phospholipase of this invention may be derived from a fungal strain of the genus *Hyphozyma*, a genus of yeast-like *Hyphomycetes* described in de Hoog, G.S & Smith, M.Th., Antonie van Leeuwenhoek, 47, 339-352 (1981).

Preferably, the strain belongs to the species defined by the strain *Hyphozyma sp.* LF132, CBS 648.91, which is described in WO 93/24619. This strain was classified in the genus Hyphozyma, but it did not match any of the previously described species of *Hyphozyma*, so it is believed to define a new species. It is particularly preferred to use said strain or a mutant or variant thereof having the ability to produce phospholipase.

The preferred *Hyphozyma sp.* strain (designated LF132 by the inventors) has been deposited on 12 November 1991, for the purpose of patent procedures according to the Budapest Treaty at Centraal Bureau voor Schimmelcultures (CBS), Oosterstraat 1, 3740 AG Baarn, Netherlands, and was given the accession number CBS 648.91.

Production of phospholipase by cultivation of *Hyphozyma*

The phospholipase of the invention may be produced by cultivation of the microorganism described above in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the enzyme. The nutrient medium may be formulated according to principles well known in the art.

The phospholipase may be recovered from the culture broth and purified to remove lipase activity, e.g. as described in the examples of this specification.

Production by cultivation of transformant

An alternative method of producing the phospholipase of the invention comprises transforming a suitable host cell with a DNA sequence encoding the phospholipase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism is preferably a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, preferably a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, most preferably *A. niger*, *A. oryzae*, *F. graminearum*, *F. sambucinum*, *F. cerealis* or *S. cerevisiae*. The production of the phospholipase in such host organisms may be done by the general methods described in EP 238,023 (Novo Nordisk), WO 96/00787 (Novo Nordisk) or EP 244,234 (Alko).

The DNA sequence can be isolated from a phospholipase-producing *Hyphozyma* strain by extraction of DNA by methods known in the art, e.g. as described by Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY.

The DNA sequence of the invention can also be isolated by any general method involving

- cloning, in suitable vectors, a cDNA library from a phospholipase-producing *Hyphozyma* strain,
- transforming suitable yeast host cells with said vectors,
- culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the cDNA library,
- screening for positive clones by determining any phospholipase activity of the enzyme produced by such clones, and
- isolating the enzyme encoding DNA from such clones.

A general isolation method has been disclosed in WO 93/11249 or WO 94/14953, the contents of which are hereby incorporated by reference.

Alternatively, the DNA encoding a phospholipase of the invention may, in accordance with well-known procedures, conveniently be isolated from a phospholipase-producing *Hyphozyma* strain, by use of synthetic oligonucleotide probes prepared on the basis of a peptide sequence disclosed herein.

Use of phospholipase

The phospholipase of the invention can be used in any application where it is desired to hydrolyze the fatty acyl group(s) of a phospholipid or lyso-phospholipid, such as lecithin or lyso-lecithin. The phospholipase is preferably used at pH 1.5-5 (e.g. 3-5, particularly 3.5-4.5) and at 30-70°C (particularly 40-60°C). If desired, the phospholipase may be inactivated after the reaction by a heat treatment, e.g. at pH 7, 80°C for 1 hour or 90°C for 10 minutes.

As an example, the phospholipase of the invention can be used in the preparation of dough, bread and cakes, e.g. to improve the elasticity of the bread or

cake. Thus, the phospholipase can be used in a process for making bread, comprising adding the phospholipase to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy with US 4,567,046 (Kyowa Hakko), JP-A 60-78529 (QP Corp.), JP-A 62-111629 (QP Corp.), JP-A 63-258528 (QP Corp.) or EP 426211 (Unilever).

The phospholipase of the invention can also be used to improve the filterability of an aqueous solution or slurry of carbohydrate origin by treating it with the phospholipase. This is particularly applicable to a solution or slurry containing a starch hydrolysate, especially a wheat starch hydrolysate since this tends to be difficult to filter and to give cloudy filtrates. The treatment can be done in analogy with EP 219,269 (CPC International).

Treatment of vegetable oil

The phospholipase of the invention can be used in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the phospholipase so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. This process is applicable to the purification of any edible oil which contains phospholipid, e.g. vegetable oil such as soy bean oil, rape seed oil and sunflower oil.

Prior to the enzymatic treatment, the vegetable oil is preferably pretreated to remove slime (mucilage), e.g. by wet refining. Typically, the oil will contain 50-250 ppm of phosphorus as phospholipid at the start of the treatment with phospholipase, and the process of the invention can reduce this value to below 5-10 ppm.

The enzymatic treatment is conducted by dispersing an aqueous solution of the phospholipase, preferably as droplets with an average diameter below 10 μ(micro)m. The amount of water is preferably 0.5-5% by weight in relation to the oil. An emulsifier may optionally be added. Mechanical agitation may be applied to maintain the emulsion.

The enzymatic treatment can be conducted at a pH in the range 1.5-5. The process pH may be in the range 3.5-5 in order to maximize the enzyme performance, or a pH in the range 1.5-3 (e.g. 2-3) may be used in order to suppress the alkaline hydrolysis of triglycerides (saponification). The pH may be adjusted by adding citric acid, a citrate buffer or HCl.

A suitable temperature is generally 30-70°C (particularly 30-45°C, e.g. 35-40°C). The reaction time will typically be 1-12 hours (e.g. 2-6 hours), and a suitable enzyme dosage will usually be 100-5000 IU per liter of oil (e.g. 200-2000 IU/l) or 0.1-10 mg/l (e.g. 0.5-5 mg/l).

The enzymatic treatment may be conducted batchwise, e.g. in a tank with stirring, or it may be continuous, e.g. a series of stirred tank reactors.

The enzymatic treatment is followed by separation of an aqueous phase and an oil phase. This separation may be performed by conventional means, e.g. centrifugation. The aqueous phase will contain phospholipase, and the enzyme may be re-used to improve the process economy.

In other respects, the process can be conducted according to principles known in the art, e.g. in analogy with US 5,264,367 (Metallgesellschaft, Röhm); K. Dahlke & H. Buchold, INFORM, 6 (12), 1284-91 (1995); H. Buchold, Fat Sci. Technol., 95 (8), 300-304 (1993); JP-A 2-153997 (Showa Sangyo); or EP 654,527 (Metallgesellschaft, Röhm).

EXAMPLES

Example 1

Production of phospholipase by cultivation of *Hyphozyma*

The strain *Hyphozyma sp.* CBS 648.91, was cultivated in a nutrient medium containing the following components:

Glucose	20 g/l
Peptone	10 g/l
$MgSO_4$, 7 H_2O	1 g/l
Yeast Extract	10 g/l
K_2HPO_4	5 g/l

pH adjusted to 6.5 with NaOH

The strain was cultivated at 27-30 C for 3-4 days. The culture broth was subjected to liquid/solid separation by centrifugation. After centrifugation, a phospholipase activity of 1 unit/g culture broth was obtained (unit defined above). The supernatant was desalted and freeze-dried resulting in a crude powder preparation.

Example 2

Purification of phospholipase

Freeze dried phospholipase powder obtained according to Example 1 (300 units/g) was applied on a Butyl Toyopearl 650M column after adjusting the salt concentration to 3.5 M ammonium acetate. Bound phospholipase activity was eluted with H_2O and separated from lipase activity which was also present in the crude powder preparation.

Fractions containing phospholipase activity were pooled, concentrated and dialyzed. The concentrated preparation was treated by anion exchange column chromatography using DEAE Toyopearl 650M. The adsorption condition was pH 7.5 (50 mM Tris-HCl) and elution was carried out by a linear gradient of 0-0.5M NaCl.

The last step was gel filtration column chromatography using HiLoad 26/60 Superdex 200pg. The condition was 50 mM Tris-HCl pH 7.5 including 0.5M NaCl. The resulting purified phospholipase was used in the following examples.

Example 3

Characterization of phospholipase

The molecular weight (MW) of the phospholipase was found to be about 94 kDa on SDS PAGE and 87 kDa by gel filtration column chromatography. The polypeptide is believed to be glycosylated. The pI is around 5.6 on IEF PAGE.

The temperature profile was determined at pH 3.0 and 4.0 in a range of 40 to 70°C. The phospholipase was incubated for 10 minutes, and the activity was determined by the method described above. The temperature profile is presented in Fig. 1 as relative activity (taking the maximum activity as 100%). It is seen from this figure that both at pH 3 and 4, the phospholipase has high activity (more than 50% of optimum) at temperatures of 40 to 60°C with a temperature optimum around 50°C.

The pH profile was determined at 40°C using glycine-HCl buffer at pH 2, 2,5 and 3, and citrate buffer at pH 3, 4, 5 and 6. The results are presented in Fig. 2 as relative activity (taking the maximum activity as 100%). Due to a change of buffer system (glycine-HCl, citrate), the figure is made up of two curves, one representing the interval of pH 2.0 to 3.0 and the other representing the interval of pH 3.0 to 6.0. From the figure it appears that the phospholipase is active at pH values of 2 to 5, and the pH optimum is around 3.

The thermostability was determined by incubating in 0.1 M phosphate buffer (pH 7) for 10 minutes at temperatures of 40-80°C and determining the residual activity after the incubation. The results were 100% at 40°C, 95% at 50°C, 82% at 60°C, 55% at 70°C and 9% at 80°C. These results are also shown in Fig. 3.

Example 4

Hydrolysis of phospholipid

A substrate solution was prepared by dissolving 2% of crude soy bean lecithin (phosphatidyl choline) in water. An enzyme solution was prepared by 50 times dilution of the purified enzyme from Example 2. 0.5 ml of the substrate solution, 0.25 ml of 0.4 M citrate buffer (pH 4) and 0.05 ml of 0.1 N $CaCl_2$ were mixed and incubated at 60°C.

0.1 ml of the enzyme solution was added and incubated for 1 hour at 60°C. The reaction was terminated by adding 0.1 ml of 1 N HCl. The mixture after the reaction was analyzed by TLC-Iatroscan as described above in the assay for reaction pattern.

The results showed that fatty acid was formed and that no lecithin remained after the reaction. A solid precipitate was observed at the bottom of the reaction vessel. This was believed to be a mixture of phospholipid and fatty acid.

Example 5

Hydrolysis of lyso-phospholipid

Lyso-phosphatidylcholine (LPC) was treated for 10 minutes at 40°C, other conditions being the same as described in Example 4. The chromatogram showed that about two thirds of the LPC was hydrolyzed, and that fatty acid was formed together with a small amount of phosphatidylcholine.

Example 6

Enzymatic degumming of edible oil

Vegetable oil was degummed by treating it with phospholipase from *Hyphozyma* as follows. The enzyme dosage, the reaction pH and temperature were varied, and the resulting content of phospholipid was measured.

The equipment consisted of a 1 l jacketed steel reactor fitted with a steel lid, a propeller (600 rpm), baffles, a temperature sensor, an inlet tube at the top, a reflux condenser (4 °C) at the top, and an outlet tube at the bottom. The reactor jacket was connected to a thermostat bath. The outlet tube was connected via silicone tubing to an in-line mixer head equipped with a high shear screen (8500 rpm, flow ca. 1.1 l/minute).The mixer head was fitted with a cooling coil (5-10 °C) and an outlet tube, which was connected to the inlet tube of the reactor via silicone tubing. A temperature sensor was inserted in the silicone tubing just after the mixer head. The only connection from the reactor/mixer head system to the atmosphere was through the reflux condenser.

In each experiment, 0.6 l (ca. 560 g) of water-degummed rape seed oil with a P content of 186-252 ppm was loaded into the reactor with the thermostat and lab mixer running and pre-treated for 30 minutes with 0.6 g (2.86 mmol) of citric acid monohydrate in 27 g of water (added water vs. oil equals 4.8% w/w; [citric acid] in water phase = 106 mM, in water/oil emulsion = 4.6 mM) at time= 0. After the pre-treatment, the pH was adjusted by adding a NaOH solution followed by the enzyme solution. The mixture was then incubated for 6 hours, and samples for P-analysis and pH determination were drawn at intervals throughout the experiment.

The determination of phosphorous content in the oil was done according to procedure 2.421 in "Standard Methods for the Analysis of Oils, Fats, and Derivatives, 7.th ed. (1987)" after separating the emulsion by heating and centrifugation.

The initial performance was calculated from the initial rate of phosphorus removal from the oil, taking the optimum as 100 %.

Degumming at various pH

The oil was treated at 40°C with an enzyme dosage of 1.3 mg/kg oil (as pure enzyme protein). The results at various pH were as follows:

pH	Initial performance (relative to optimum)	P content after 6 hours
3.0	40	74 ppm
3.7	90	<10 ppm
4.4	100	<10 ppm
4.8	80	<10 ppm

Degumming at varioustemperatures

The oil was treated at pH 4.5 with an enzyme dosage of 1.3 mg/kg oil (as pure enzyme protein). The results at various temperatures were as follows:

Temperature	Initial performance (relative to optimum)	P content after 6 hours
35°C	90	<10 ppm
40°C	100	<10 ppm

Degumming with various enzyme dosages

The oil was treated at pH 4.5, 40°C. The results at various enzyme dosages (given as pure enzyme protein) were as follows:

Enzyme dosage	Initial performance (relative to optimum)	P content after 6 hours
0.65 mg/kg oil	70	<10 ppm
1.3	100	<10 ppm
2.6	100	<10 ppm

The results show good degumming performance at pH 3.5-5, 35-40°C. Good degumming to a phosphorus content below 10 ppm was obtained in 6 hours with a dosage of 1.3 mg/kg oil, and in 3 hours at a dosage of 2.6 mg/kg.

Measurement of the free fatty acids generated during degumming showed a low level of free fatty acids, corresponding very well to the amount of phospholipid in the substrate oil.

For reference, similar experiments were done with prior-art phospholipase from porcine pancreas. It was found that degumming to below 10 ppm of phosphorus could be obtained at 60°C, pH 5.5, but the performance of the prior-art enzyme dropped sharply at lower pH, and satisfactory degumming could not be achieved at pH lower than 5.5.

Example 7

Partial determination of the DNA sequence encoding the phospholipase

DNA encoding the phospholipase of *Hyphozyma* was isolated by two different methods. The 5' end of the gene was isolated by cloning. A genomic library of *Hyphozyma* DNA partially digested with Sau3A was screened at high stringency using standard methods (Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY) with a probe specific to the phospholipase sequence. This probe was amplified from total *Hyphozyma* DNA with degenerate primers designed using the previously determined partial peptide sequences with SEQ ID NO: 1 and 5. Standard PCR conditions were used for amplification (Saiki el al., Science, 239, 487-491, 1988), including 0.5mM $MgCl_2$, a 45°C annealing temperature, and primers PLMStr1 (SEQ ID NO: 12) and PLMStr6 (SEQ ID NO: 13). The clone pMStr16 hybridized to the probe, and therefore was isolated and a portion of the insert was sequenced.

An additional internal portion of the phospholipase-encoding gene was isolated using PCR with *Hyphozyma* DNA and the primers PLHaW2 (SEQ ID NO: 14) and PLMStr7 (SEQ ID NO: 15). PLHaW2 was designed using the sequence determined from pMStr16, and PLMStr7 was designed from the sequence of the partial peptide with SEQ ID NO: 8. Standard conditions were used for the PCR reactions, with 1.5 mM $MgCl_2$, and a 46°C annealing temperature. The resulting amplified fragment was isolated and sequenced.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Novo Nordisk A/S

(B) STREET: Novo Alle

(C) CITY: Bagsvaerd

(E) COUNTRY: Denmark

(F) POSTAL CODE (ZIP): DK-2880

(G) TELEPHONE: +45-4444-8888

(I) TELEX: +45-4449-3256

(ii) TITLE OF INVENTION: Novel Phospholipase, Production and Use Thereof

(iii) NUMBER OF SEQUENCES: 15

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hyphozyma sp.

(B) STRAIN: CBS 648.91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Ser Pro Ser Gly Ser Tyr Ala Pro Ala Asn Met Pro Cys Xaa Gln
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hyphozyma sp.

(B) STRAIN: CBS 648.91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Asp Trp Ala Lys Trp Leu Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hyphozyma sp.

(B) STRAIN: CBS 648.91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Asp Gly Arg Xaa Glu Thr Ala Asn Gln Arg Gly Thr Gly Gly Leu Leu
1 5 10 15

Gln Leu Ala Glu Tyr Ile Ala Gly Leu Ser Gly Gly
20 25

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hyphozyma sp.

(B) STRAIN: CBS 648.91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Asp Leu Glu Ser Asn Leu Ile Val Pro Glu Asp Gly Lys Val Ser Phe
1 5 10 15

Tyr Ala Ser Ile Leu Ala Ala Val Ala Gly Lys Arg Asn Glu Gly Tyr
20 25 30

Gln Thr Ser Leu
35

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hyphozyma sp.

(B) STRAIN: CBS 648.91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Asp Glu Arg Glu Pro Gly Glu Leu Ile Ile Pro Arg Xaa Thr Thr Ile
1 5 10 15

Trp Glu Phe Asn Pro Tyr Glu Phe Gly Ser Trp Asn Pro Xaa Val Ser
20 25 30

Ala Phe Ile Pro Ile Glu Ile Leu Gly
35 40

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hyphozyma sp.

(B) STRAIN: CBS 648.91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Asp Val Ser Leu Val Pro Asn Pro Phe Tyr Gly Tyr Val Gly Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hyphozyma sp.
(B) STRAIN: CBS 648.91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Asp Val Thr Asn Trp Pro Xaa Ala Ser Ala Leu Tyr Gln Thr Ser Leu
1 5 10 15

Arg Ala Gln Tyr Pro Thr Tyr Ser Gln Tyr Ala Phe Pro Val
20 25 30

(2) INFORMATION FOR SEQ ID NO: 8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Hyphozyma sp.
(B) STRAIN: CBS 648.91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Asp Thr Ser Phe Xaa Gly Thr Lys Thr Pro Ile Ile Val Tyr Met Pro
1 5 10 15

Ser Tyr Pro Tyr Ala Ala Phe Ala Asp Thr Ser Thr Phe Lys Leu
20 25 30

(2) INFORMATION FOR SEQ ID NO: 9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1870 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hyphozyma sp.

(B) STRAIN: CBS 648.91

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:214..1869

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION:442..1869

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
GGCGAGTGCA CAAGGCCGCG GACCAAATGT CCCTGAGTGC GTGTGTTTGT GTGTGACATA      60

GCCAGCAGAA TGCAGCTTAC TCTTCTTCCA TTGTGAGACG TTATATACCC ACACACATCT     120

CGCCGTCCCG TCAGACCCTT CTGCATCCGT CCGTACGAAC CTGCTCTCTT CCATTTACCT     180

CGACACTGTA TCGAGTGCAC GCTTCGAGGC ATC ATG AAG CTG CCG CTC CTC TCT      234
                                     Met Lys Leu Pro Leu Leu Ser
                                     -76 -75                 -70

ACG CTG CTC AGC CTC GCG CTG ACC GCC TCG ACC GTC GTC CGT GCC TAT      282
Thr Leu Leu Ser Leu Ala Leu Thr Ala Ser Thr Val Val Arg Ala Tyr
               -65                 -60                 -55

CCC TCC ATC CCG GCG CAG CTC ACC GAA GAC GAG ATC ACC CGC ATC AGC      330
Pro Ser Ile Pro Ala Gln Leu Thr Glu Asp Glu Ile Thr Arg Ile Ser
          -50                 -45                 -40
```

```
CAG CTC TCC CAG GAG GAC AAG GTC AAG TTT GCC GAA CGC ATC CTA GAG      378
Gln Leu Ser Gln Glu Asp Lys Val Lys Phe Ala Glu Arg Ile Leu Glu
        -35                 -30                 -25

ATT CGC ACC GCC TAC GAG TAT GAG AAG CAG CAG CTA GCC CGT CAA CAT      426
Ile Arg Thr Ala Tyr Glu Tyr Glu Lys Gln Gln Leu Ala Arg Gln His
    -20                 -15                 -10

GCG CTC GAG CGA CGC GCC TCG CCC TCG GGC TCG TAC GCA CCT GCC AAC      474
Ala Leu Glu Arg Arg Ala Ser Pro Ser Gly Ser Tyr Ala Pro Ala Asn
 -5                  1                   5                  10

ATG CCC TGC CCC CAG CGA ACG TCC CAG CAG GGT CCC GGC TTC ATC CGA      522
Met Pro Cys Pro Gln Arg Thr Ser Gln Gln Gly Pro Gly Phe Ile Arg
            15                  20                  25

CCC GCC AAG ACC AAG CAG CTC TCA ATC TCG GAA GCC GAC TAT GTC TCG      570
Pro Ala Lys Thr Lys Gln Leu Ser Ile Ser Glu Ala Asp Tyr Val Ser
        30                  35                  40

CGC CGC CGC ACC AAC ACC CAG GCC GAC TGG GCC AAG TGG CTC TCG GAC      618
Arg Arg Arg Thr Asn Thr Gln Ala Asp Trp Ala Lys Trp Leu Ser Asp
    45                  50                  55

TCG GCC AAG CTC AAC AGC AGC CTG CCC GGC GGT GCC TCC AAC TAC ACC      666
Ser Ala Lys Leu Asn Ser Ser Leu Pro Gly Gly Ala Ser Asn Tyr Thr
60                  65                  70                  75

TCG TCG ACC GAC CGC GTG CCT CGT CTG GGC TTT GCG CTC AGC GGC GGT      714
Ser Ser Thr Asp Arg Val Pro Arg Leu Gly Phe Ala Leu Ser Gly Gly
                80                  85                  90

GGA CTG CGT GCC ATG CTC GTT GGT TCG GGC ACG CTC CAG GGC TTT GAC      762
Gly Leu Arg Ala Met Leu Val Gly Ser Gly Thr Leu Gln Gly Phe Asp
            95                  100                 105
```

```
GGC CGC AAC GAG ACC GCC AAC CAG CGT GGC ACC GGT GGA CTG CTC CAG        810
Gly Arg Asn Glu Thr Ala Asn Gln Arg Gly Thr Gly Gly Leu Leu Gln
        110                 115                 120

CTT GCC GAG TAC ATT GCC GGC CTG TCC GGC GGC TCG TGG GCG ACC GCC        858
Leu Ala Glu Tyr Ile Ala Gly Leu Ser Gly Gly Ser Trp Ala Thr Ala
    125                 130                 135

AGT CTC ACC ATG AAC AAC TGG GCC ACC ACC CAG TCG CTC AAG GAC AAC        906
Ser Leu Thr Met Asn Asn Trp Ala Thr Thr Gln Ser Leu Lys Asp Asn
140                 145                 150                 155

ATC TGG GAT CTC GAG TCC AAC CTC ATC GTC CCC GAG GAC GGC AAG GTC        954
Ile Trp Asp Leu Glu Ser Asn Leu Ile Val Pro Glu Asp Gly Lys Val
                160                 165                 170

TCG TTT TAC GCC TCG ATC CTG GCC GCC GTC GCG GGC AAG AGG AAC GAA       1002
Ser Phe Tyr Ala Ser Ile Leu Ala Ala Val Ala Gly Lys Arg Asn Glu
            175                 180                 185

GGT TAC CAG ACC AGT CTC ACC GAC TAC TTT GGC CTC TCG ATC GCC GAC       1050
Gly Tyr Gln Thr Ser Leu Thr Asp Tyr Phe Gly Leu Ser Ile Ala Asp
        190                 195                 200

AAG ATT CTC AAC GGC TCC ATG TAC GGC AAC AAG TTC AGC GTC GAG TGG       1098
Lys Ile Leu Asn Gly Ser Met Tyr Gly Asn Lys Phe Ser Val Glu Trp
    205                 210                 215

AGC GAC GTC AAG AAT ACG TCC AAG TTC ACC GAT GCC TCC ATG CCG TTC       1146
Ser Asp Val Lys Asn Thr Ser Lys Phe Thr Asp Ala Ser Met Pro Phe
220                 225                 230                 235

CCC ATC ATT ATT GCC GAC GAG CGC GAG CCC GGC GAG CTC ATC ATC CCG       1194
Pro Ile Ile Ile Ala Asp Glu Arg Glu Pro Gly Glu Leu Ile Ile Pro
                240                 245                 250
```

```
CGC AAC ACC ACC ATC TGG GAG TTC AAC CCG TAC GAG TTC GGT TCT TGG      1242
Arg Asn Thr Thr Ile Trp Glu Phe Asn Pro Tyr Glu Phe Gly Ser Trp
            255                 260                 265

AAC CCC AAT GTT TCG GCT TTC ATC CCC ATC GAG ATC CTC GGC TCG AGT      1290
Asn Pro Asn Val Ser Ala Phe Ile Pro Ile Glu Ile Leu Gly Ser Ser
        270                 275                 280

CTG GAC AAC GGC ACC AGC GTC CTG CCC GAC GGC GTC TGT GTC GGC GGA      1338
Leu Asp Asn Gly Thr Ser Val Leu Pro Asp Gly Val Cys Val Gly Gly
    285                 290                 295

TAC GAG ACC GTT GCC TGG GTG ACT GGC ACC TCG GCG ACT CTG TTC TCT      1386
Tyr Glu Thr Val Ala Trp Val Thr Gly Thr Ser Ala Thr Leu Phe Ser
300                 305                 310                 315

GGT CTG TAC CTC GAA CTT ATC TCG ACC TCG AGC AAC AAC ATC ATC GTC      1434
Gly Leu Tyr Leu Glu Leu Ile Ser Thr Ser Ser Asn Asn Ile Ile Val
                320                 325                 330

GAT GCG CTC AAG GAG ATT GCC CAG GCG GTA TCA AAC GAG CAG AAC GAT      1482
Asp Ala Leu Lys Glu Ile Ala Gln Ala Val Ser Asn Glu Gln Asn Asp
            335                 340                 345

GTC TCG CTC GTG CCC AAC CCG TTC TAC GGC TAC GTC GGC GAA GGC GAC      1530
Val Ser Leu Val Pro Asn Pro Phe Tyr Gly Tyr Val Gly Glu Gly Asp
        350                 355                 360

GTC CAA GTG TCG GAC CTG CGC AAT ATT ACG CTC GTC GAT GGT GGT CTC      1578
Val Gln Val Ser Asp Leu Arg Asn Ile Thr Leu Val Asp Gly Gly Leu
    365                 370                 375

GAC AAC GAG AAT GTG CCA CTC TGG CCG CTT GTC GAG CCG GCG CGC GAT      1626
Asp Asn Glu Asn Val Pro Leu Trp Pro Leu Val Glu Pro Ala Arg Asp
380                 385                 390                 395
```

```
CTG GAC GTG ATC ATC GCC ATT GAC AGC TCG GCG GAC GTG ACC AAC TGG      1674
Leu Asp Val Ile Ile Ala Ile Asp Ser Ser Ala Asp Val Thr Asn Trp
                400                 405                 410

CCG AAC GCG TCG GCG CTG TAC CAG ACG TCG CTG CGT GCT CAG TAC CCG      1722
Pro Asn Ala Ser Ala Leu Tyr Gln Thr Ser Leu Arg Ala Gln Tyr Pro
            415                 420                 425

ACC TAT AGC CAG TAC GCG TTC CCG GTG ATG CCG GAC ACC AAC ACG GTG      1770
Thr Tyr Ser Gln Tyr Ala Phe Pro Val Met Pro Asp Thr Asn Thr Val
        430                 435                 440

GTC AAC CGC GGC CTC AAC ACG CGC CCC GTG TTC TAC GGC TGC AAT GCG      1818
Val Asn Arg Gly Leu Asn Thr Arg Pro Val Phe Tyr Gly Cys Asn Ala
    445                 450                 455

ACC GTC AAC GTC ACC AAC GCG GAT ACG TCG TTC AAC GGC ACC AAG ACG      1866
Thr Val Asn Val Thr Asn Ala Asp Thr Ser Phe Asn Gly Thr Lys Thr
460                 465                 470                 475

CCA A                                                               1870
Pro
```

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 552 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

```
Met Lys Leu Pro Leu Leu Ser Thr Leu Leu Ser Leu Ala Leu Thr Ala
-76 -75                 -70                 -65

Ser Thr Val Val Arg Ala Tyr Pro Ser Ile Pro Ala Gln Leu Thr Glu
-60                 -55                 -50                 -45
```

```
Asp Glu Ile Thr Arg Ile Ser Gln Leu Ser Gln Glu Asp Lys Val Lys
                -40                 -35                 -30

Phe Ala Glu Arg Ile Leu Glu Ile Arg Thr Ala Tyr Glu Tyr Glu Lys
            -25                 -20                 -15

Gln Gln Leu Ala Arg Gln His Ala Leu Glu Arg Arg Ala Ser Pro Ser
        -10                 -5                  1

Gly Ser Tyr Ala Pro Ala Asn Met Pro Cys Pro Gln Arg Thr Ser Gln
  5                 10                  15                  20

Gln Gly Pro Gly Phe Ile Arg Pro Ala Lys Thr Lys Gln Leu Ser Ile
                25                  30                  35

Ser Glu Ala Asp Tyr Val Ser Arg Arg Arg Thr Asn Thr Gln Ala Asp
            40                  45                  50

Trp Ala Lys Trp Leu Ser Asp Ser Ala Lys Leu Asn Ser Ser Leu Pro
        55                  60                  65

Gly Gly Ala Ser Asn Tyr Thr Ser Ser Thr Asp Arg Val Pro Arg Leu
    70                  75                  80

Gly Phe Ala Leu Ser Gly Gly Gly Leu Arg Ala Met Leu Val Gly Ser
85                  90                  95                  100

Gly Thr Leu Gln Gly Phe Asp Gly Arg Asn Glu Thr Ala Asn Gln Arg
                105                 110                 115

Gly Thr Gly Gly Leu Leu Gln Leu Ala Glu Tyr Ile Ala Gly Leu Ser
            120                 125                 130

Gly Gly Ser Trp Ala Thr Ala Ser Leu Thr Met Asn Asn Trp Ala Thr
        135                 140                 145
```

Thr Gln Ser Leu Lys Asp Asn Ile Trp Asp Leu Glu Ser Asn Leu Ile
150 155 160

Val Pro Glu Asp Gly Lys Val Ser Phe Tyr Ala Ser Ile Leu Ala Ala
165 170 175 180

Val Ala Gly Lys Arg Asn Glu Gly Tyr Gln Thr Ser Leu Thr Asp Tyr
185 190 195

Phe Gly Leu Ser Ile Ala Asp Lys Ile Leu Asn Gly Ser Met Tyr Gly
200 205 210

Asn Lys Phe Ser Val Glu Trp Ser Asp Val Lys Asn Thr Ser Lys Phe
215 220 225

Thr Asp Ala Ser Met Pro Phe Pro Ile Ile Ile Ala Asp Glu Arg Glu
230 235 240

Pro Gly Glu Leu Ile Ile Pro Arg Asn Thr Thr Ile Trp Glu Phe Asn
245 250 255 260

Pro Tyr Glu Phe Gly Ser Trp Asn Pro Asn Val Ser Ala Phe Ile Pro
265 270 275

Ile Glu Ile Leu Gly Ser Ser Leu Asp Asn Gly Thr Ser Val Leu Pro
280 285 290

Asp Gly Val Cys Val Gly Gly Tyr Glu Thr Val Ala Trp Val Thr Gly
295 300 305

Thr Ser Ala Thr Leu Phe Ser Gly Leu Tyr Leu Glu Leu Ile Ser Thr
310 315 320

Ser Ser Asn Asn Ile Ile Val Asp Ala Leu Lys Glu Ile Ala Gln Ala
325 330 335 340

```
Val Ser Asn Glu Gln Asn Asp Val Ser Leu Val Pro Asn Pro Phe Tyr
                345                 350                 355

Gly Tyr Val Gly Glu Gly Asp Val Gln Val Ser Asp Leu Arg Asn Ile
            360                 365                 370

Thr Leu Val Asp Gly Gly Leu Asp Asn Glu Asn Val Pro Leu Trp Pro
        375                 380                 385

Leu Val Glu Pro Ala Arg Asp Leu Asp Val Ile Ile Ala Ile Asp Ser
    390                 395                 400

Ser Ala Asp Val Thr Asn Trp Pro Asn Ala Ser Ala Leu Tyr Gln Thr
405                 410                 415                 420

Ser Leu Arg Ala Gln Tyr Pro Thr Tyr Ser Gln Tyr Ala Phe Pro Val
                425                 430                 435

Met Pro Asp Thr Asn Thr Val Val Asn Arg Gly Leu Asn Thr Arg Pro
            440                 445                 450

Val Phe Tyr Gly Cys Asn Ala Thr Val Asn Val Thr Asn Ala Asp Thr
        455                 460                 465

Ser Phe Asn Gly Thr Lys Thr Pro
    470                 475
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 573 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hyphozyma sp.

(B) STRAIN: CBS 648.91

(ix) FEATURE:

(A) NAME/KEY: Protein

(B) LOCATION:1..497

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
Met Lys Leu Pro Leu Leu Ser Thr Leu Leu Ser Leu Ala Leu Thr Ala
    -75                 -70                 -65

Ser Thr Val Val Arg Ala Tyr Pro Ser Ile Pro Ala Gln Leu Thr Glu
-60                 -55                 -50                 -45

Asp Glu Ile Thr Arg Ile Ser Gln Leu Ser Gln Glu Asp Lys Val Lys
                -40                 -35                 -30

Phe Ala Glu Arg Ile Leu Glu Ile Arg Thr Ala Tyr Glu Tyr Glu Lys
            -25                 -20                 -15

Gln Gln Leu Ala Arg Gln His Ala Leu Glu Arg Arg Ala Ser Pro Ser
        -10                 -5                  1

Gly Ser Tyr Ala Pro Ala Asn Met Pro Cys Pro Gln Arg Thr Ser Gln
5                   10                  15                  20

Gln Gly Pro Gly Phe Ile Arg Pro Ala Lys Thr Lys Gln Leu Ser Ile
                25                  30                  35

Ser Glu Ala Asp Tyr Val Ser Arg Arg Arg Thr Asn Thr Gln Ala Asp
            40                  45                  50

Trp Ala Lys Trp Leu Ser Asp Ser Ala Lys Leu Asn Ser Ser Leu Pro
        55                  60                  65

Gly Gly Ala Ser Asn Tyr Thr Ser Ser Thr Asp Arg Val Pro Arg Leu
    70                  75                  80
```

Gly Phe Ala Leu Ser Gly Gly Gly Leu Arg Ala Met Leu Val Gly Ser
85 90 95 100

Gly Thr Leu Gln Gly Phe Asp Gly Arg Asn Glu Thr Ala Asn Gln Arg
105 110 115

Gly Thr Gly Gly Leu Leu Gln Leu Ala Glu Tyr Ile Ala Gly Leu Ser
120 125 130

Gly Gly Ser Trp Ala Thr Ala Ser Leu Thr Met Asn Asn Trp Ala Thr
135 140 145

Thr Gln Ser Leu Lys Asp Asn Ile Trp Asp Leu Glu Ser Asn Leu Ile
150 155 160

Val Pro Glu Asp Gly Lys Val Ser Phe Tyr Ala Ser Ile Leu Ala Ala
165 170 175 180

Val Ala Gly Lys Arg Asn Glu Gly Tyr Gln Thr Ser Leu Thr Asp Tyr
185 190 195

Phe Gly Leu Ser Ile Ala Asp Lys Ile Leu Asn Gly Ser Met Tyr Gly
200 205 210

Asn Lys Phe Ser Val Glu Trp Ser Asp Val Lys Asn Thr Ser Lys Phe
215 220 225

Thr Asp Ala Ser Met Pro Phe Pro Ile Ile Ile Ala Asp Glu Arg Glu
230 235 240

Pro Gly Glu Leu Ile Ile Pro Arg Asn Thr Thr Ile Trp Glu Phe Asn
245 250 255 260

Pro Tyr Glu Phe Gly Ser Trp Asn Pro Asn Val Ser Ala Phe Ile Pro
265 270 275

Ile Glu Ile Leu Gly Ser Ser Leu Asp Asn Gly Thr Ser Val Leu Pro
280 285 290

Asp Gly Val Cys Val Gly Gly Tyr Glu Thr Val Ala Trp Val Thr Gly
295 300 305

Thr Ser Ala Thr Leu Phe Ser Gly Leu Tyr Leu Glu Leu Ile Ser Thr
310 315 320

Ser Ser Asn Asn Ile Ile Val Asp Ala Leu Lys Glu Ile Ala Gln Ala
325 330 335 340

Val Ser Asn Glu Gln Asn Asp Val Ser Leu Val Pro Asn Pro Phe Tyr
345 350 355

Gly Tyr Val Gly Glu Gly Asp Val Gln Val Ser Asp Leu Arg Asn Ile
360 365 370

Thr Leu Val Asp Gly Gly Leu Asp Asn Glu Asn Val Pro Leu Trp Pro
375 380 385

Leu Val Glu Pro Ala Arg Asp Leu Asp Val Ile Ile Ala Ile Asp Ser
390 395 400

Ser Ala Asp Val Thr Asn Trp Pro Asn Ala Ser Ala Leu Tyr Gln Thr
405 410 415 420

Ser Leu Arg Ala Gln Tyr Pro Thr Tyr Ser Gln Tyr Ala Phe Pro Val
425 430 435

Met Pro Asp Thr Asn Thr Val Val Asn Arg Gly Leu Asn Thr Arg Pro
440 445 450

Val Phe Tyr Gly Cys Asn Ala Thr Val Asn Val Thr Asn Ala Asp Thr
455 460 465

Ser Phe Asn Gly Thr Lys Thr Pro Ile Ile Val Tyr Met Pro Ser Tyr
470 475 480

Pro Tyr Ala Ala Phe Ala Asp Thr Ser Thr Phe Lys Leu
485 490 495

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

(A) NAME/KEY: modified_base

(B) LOCATION:3..18

(D) OTHER INFORMATION:/mod_base= OTHER
/note= "deoxyinosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GCNCCNGCNA AYATGCCNTG 20

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

(A) NAME/KEY: modified_base

(B) LOCATION:6

(D) OTHER INFORMATION:/mod_base= OTHER
/note= "deoxyinosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TCGTANGGGT TRAAYTCCCA 20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CCATGCTCGT TGGTTCG 17

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGCATGTAGA CGATGAT 17

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 6, lines 18-22.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

Centraal Bureau voor Schimmelcultures (CBS)

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, 3740 AG Barrn, Netherlands

Date of deposit	Accession Number
12 November 1991	CBS 648.91

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). As far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

☒ This sheet was received with the international application

Authorized officer

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

CLAIMS

1. A phospholipase which:
   a) is able to hydrolyze both fatty acyl groups in a phospholipid,
   b) is derivable from a strain of *Hyphozyma*,
   c) has a temperature optimum measured for 10 minutes at pH 3-4 of about 50°C, and
   d) has a pH optimum measured at 40°C for 10 minutes of about pH 3.

2. A phospholipase which:
   a) is able to hydrolyze both fatty acyl groups in a phospholipid, and
   b) is a polypeptide comprising an N-terminal amino acid sequence which is the sequence shown in positions 1-497 of SEQ ID NO: 11, or is at least 50 % identical therewith.

3. A phospholipase which:
   a) is able to hydrolyze both fatty acyl groups in a phospholipid, and
   b) is a polypeptide containing amino acid sequences which are at least 50% identical with the amino acid sequences shown in SEQ ID NO: 1-8.

4. The phospholipase of claim 2 or 3 wherein said identity of sequences is at least 60%, preferably at least 70%, more preferably at least 80%, and most preferably at least 90%.

5. The phospholipase of any preceding claim which is derivable from *Hyphozyma sp.* strain CBS 648.91.

6. The phospholipase of any preceding claim which is essentially free from lipase activity.

7. A DNA sequence which encodes the phospholipase of claim 2.

8. The DNA sequence of the preceding claim which comprises the sequence shown in positions 457-1870 of SEQ ID NO: 9.

9. A method of producing a phospholipase, comprising cultivation of a phospholipase-producing strain of *Hyphozyma* in a suitable nutrient medium, followed by recovery of the phospholipase.

10. The method of the preceding claim wherein the strain is *Hyphozyma sp.* strain CBS 648.91.

11. The method of claim 9 or 10 wherein the recovery comprises separation to remove lipase activity.

12. A method for producing a phospholipase, comprising:

a) isolating a DNA sequence encoding the phospholipase from a phospholipase-producing strain of *Hyphozyma*,

b) combining the DNA fragment with appropriate expression signal(s) in an appropriate vector,

c) transforming a suitable heterologous host organism with the vector,

d) cultivating the transformed host organism under conditions leading to expression of the phospholipase, and

e) recovering the phospholipase from the culture medium.

13. The method of the preceding claim, wherein the host organism is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, preferably a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, most preferably *A. niger*, *A. oryzae*, *F. graminearum*, *F. sambucinum*, *F. cerealis* or *S. cerevisiae*.

14. The method of claim 12 or 13 wherein the DNA sequence is isolated by a method comprising:

a) cloning, in suitable vectors, a cDNA library from the phospholipase-producing strain of *Hyphozyma*,

b) transforming suitable yeast host cells with said vectors,

c) cultivating the transformed yeast host cells under suitable conditions to express the phospholipase,

d) screening for positive clones by determining the phospholipase activity expressed in step (c).

15. The method of any of claims 12-14, wherein the *Hyphozyma* strain is *Hyphozyma sp.* strain CBS 648.91.

16. A process for hydrolyzing fatty acyl groups in a phospholipid or lysophospholipid, comprising treating the phospholipid or lysophospholipid with the phospholipase of any of claims 1-6.

17. The process of the preceding claim wherein the phospholipid or lysophospholipid comprises lecithin or lysolecithin.

18. The process of claim 16 or 17 wherein the treatment is conducted at pH 1.5-5 (preferably 2-4) and 30-70°C.

19. The process of any of claims 16-18, which is a process for improving the filterability of an aqueous solution or slurry of carbohydrate origin which contains phospholipid.

20. The process of the preceding claim wherein the solution or slurry contains a starch hydrolysate, particularly a wheat starch hydrolysate.

21. The process of any of claims 16-18 which is a process for making bread, comprising adding the phospholipase to the ingredients of a dough, kneading the dough and baking the dough to make the bread.

22. The process of any of claims 16-18 which is a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the phospholipase so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil.

23. A process for removing phospholipid from an edible oil, comprising:

a) treating the oil at pH 1.5-3 with a dispersion of an aqueous solution of a phospholipase having the ability to hydrolyze the intact phospholipid at said pH, so as to hydrolyze a major part of the phospholipid, and

b) separating an aqueous phase containing the hydrolyzed phospholipid from the oil.

24. The method of the preceding claim wherein the oil is treated to remove mucilage prior to the treatment with the phospholipase.

25. The method of claim 23 or 24 wherein the oil prior to the treatment with the phospholipase contains the phospholipid in an amount corresponding to 50-250 ppm as phosphorus.

26. The method of any of claims 23-25 wherein the phospholipase is the phospholipase of any of claims 1-6.

27. The process of any of claims 23-26 wherein the treatment with phospholipase is done at 30-45°C for 1-12 hours at a phospholipase dosage of 0.1-10 mg/l in the presence of 0.5-5% of water.

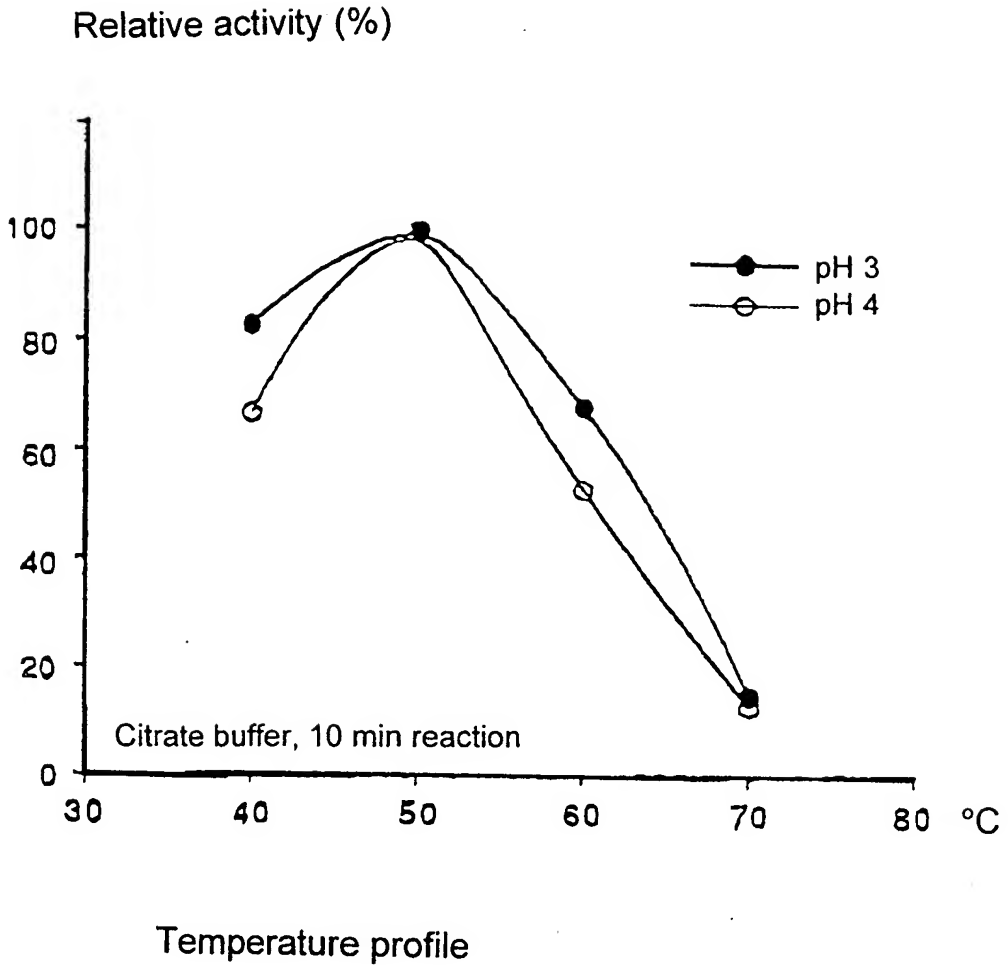
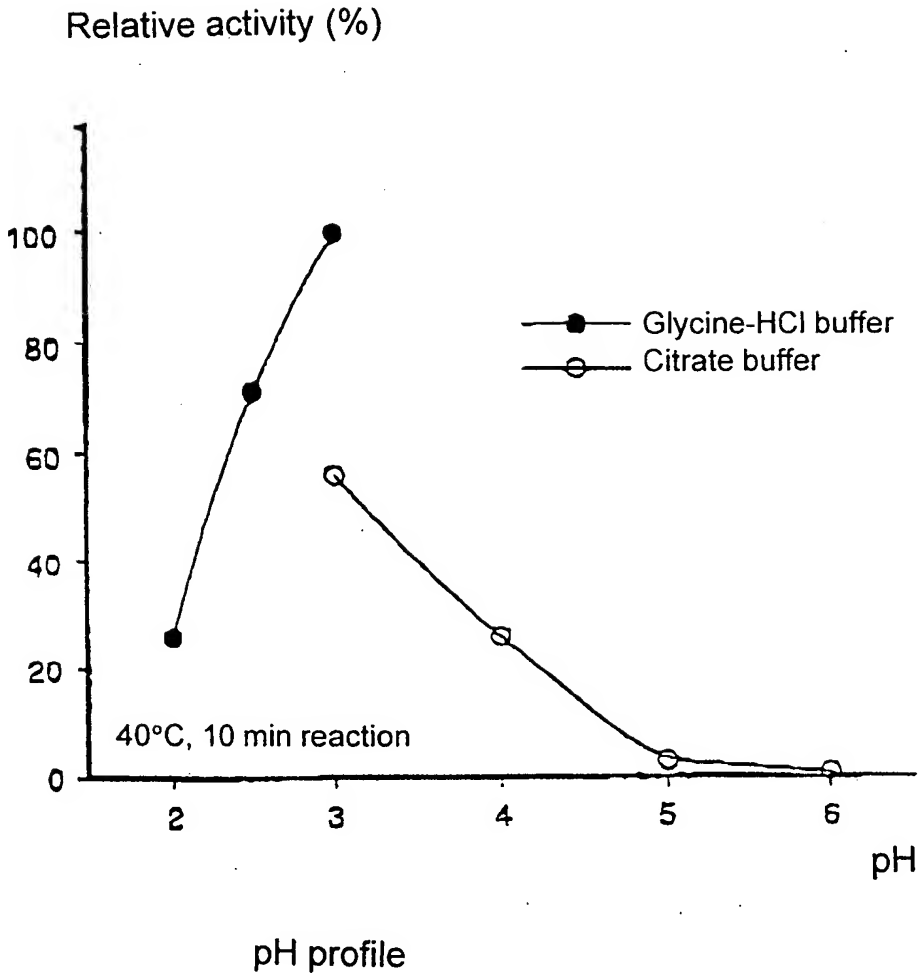

Relative activity (%)
100
80
60
40
20
0
30
40
50
60
70
80
°C
pH 3
pH 4
Citrate buffer, 10 min reaction
Temperature profile

FIG. 1



pH profile

FIG. 2

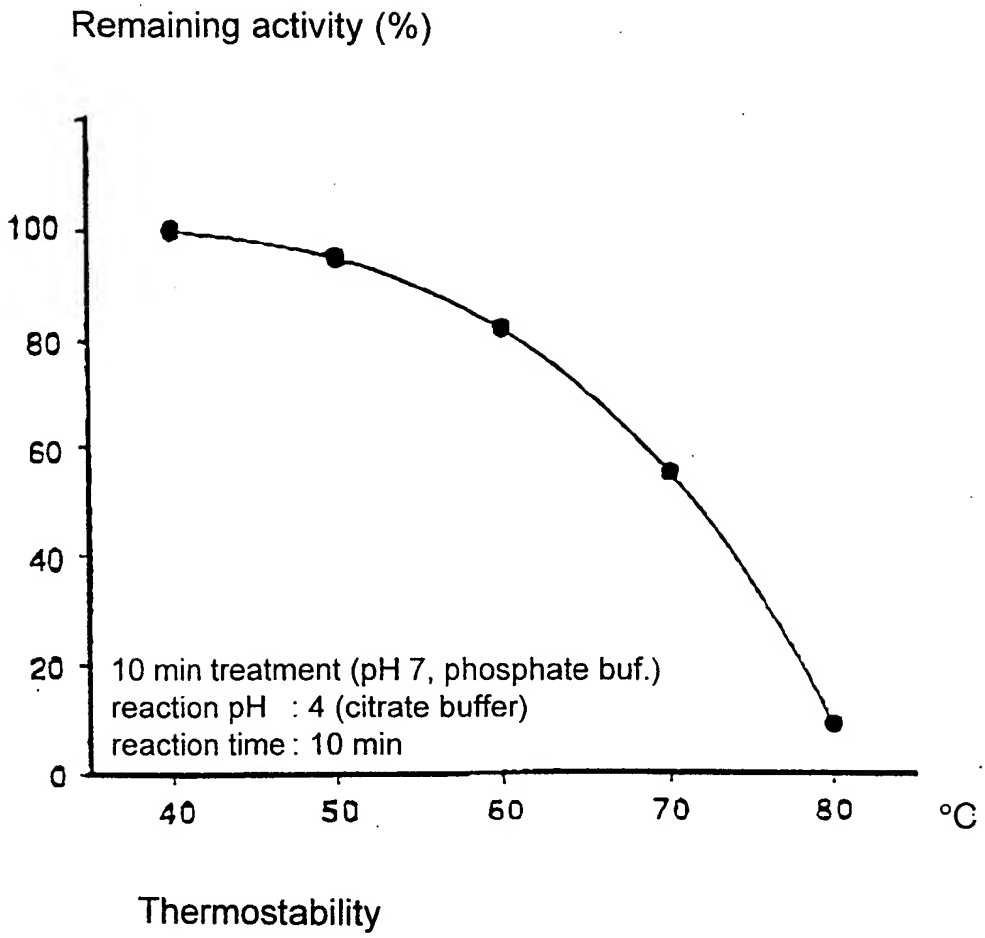

Remaining activity (%)
100
80
60
40
20
0
10 min treatment (pH 7, phosphate buf.)
reaction pH : 4 (citrate buffer)
reaction time : 10 min
40
50
60
70
80
°C
Thermostability

FIG. 3

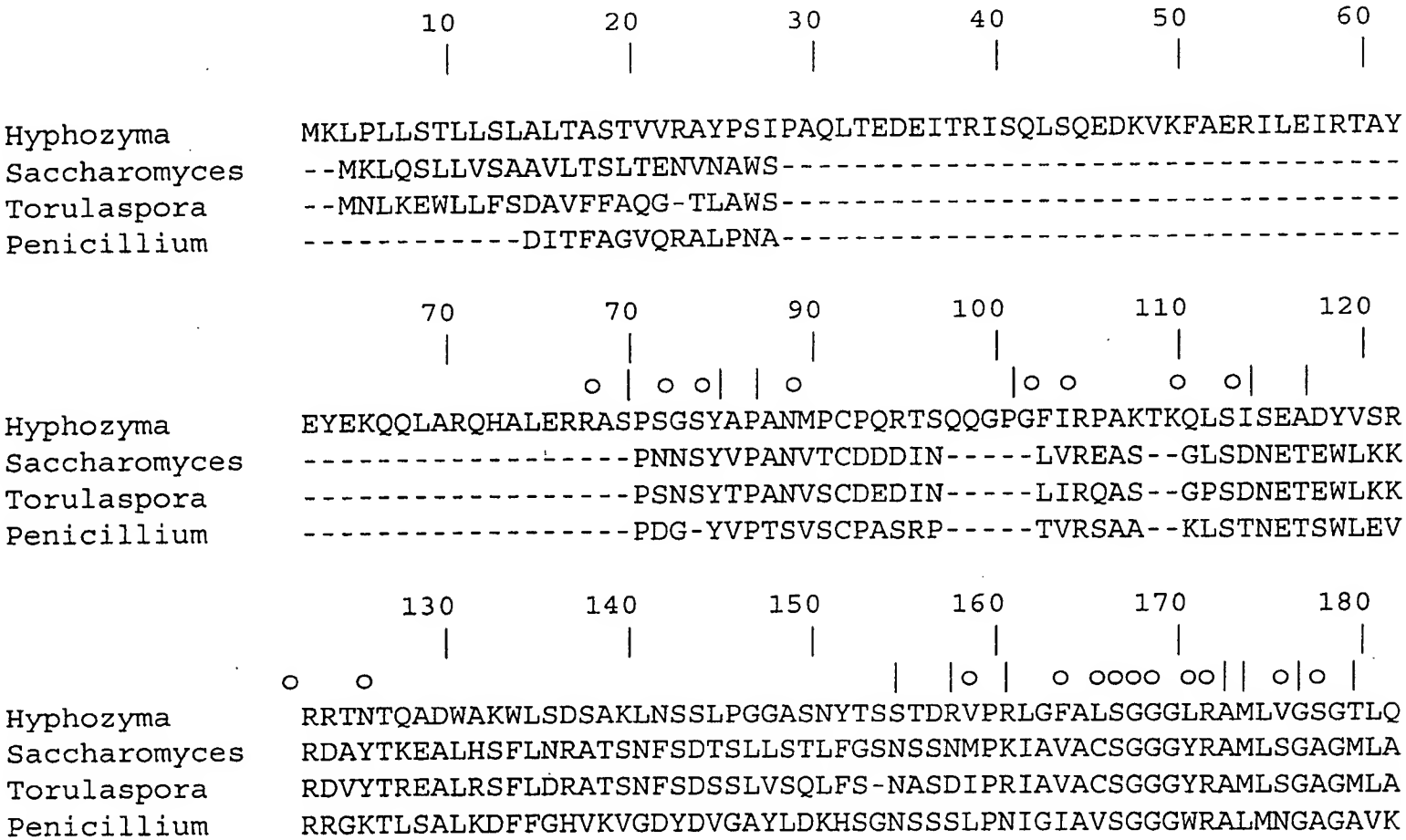

10 20 30 40 50 60
Hyphozyma MKLPLLSTLLSLALTASTVVRAYPSIPAQLTEDEITRISQLSQEDKVKFAERILEIRTAY
Saccharomyces --MKLQSLLVSAAVLTSLTENVNAWS----------
Torulaspora --MNLKEWLLFSDAVFFAQG-TLAWS----------
Penicillium ----------DITFAGVQRALPNA----------
70 70 90 100 110 120
Hyphozyma EYEKQQLARQHALERRASPSGSYAPANMPCPQRTSQQGPGFIRPAKTKQLSISEADYVSR
Saccharomyces ----------PNNSYVPANVTCDDDIN-----LVREAS--GLSDNETEWLKK
Torulaspora ----------PSNSYTPANVSCDEDIN-----LIRQAS--GPSDNETEWLKK
Penicillium ----------PDG-YVPTSVSCPASRP-----TVRSAA--KLSTNETSWLEV
130 140 150 160 170 180
Hyphozyma RRTNTQADWAKWLSDSAKLNSSLPGGASNYTSSTDRVPRLGFALSGGGLRAMLVGSGTLQ
Saccharomyces RDAYTKEALHSFLNRATSNFSDTSLLSTLFGSNSSNMPKIAVACSGGGYRAMLSGAGMLA
Torulaspora RDVYTREALRSFLDRATSNFSDSSLVSQLFS-NASDIPRIAVACSGGGYRAMLSGAGMLA
Penicillium RRGKTLSALKDFFGHVKVGDYDVGAYLDKHSGNSSSLPNIGIAVSGGGWRALMNGAGAVK

FIG. 4a

```
                190       200       210       220       230       240
                  |         |         |         |         |         |
               o o|| o|  o  ooooo | o||ooooo o   ||  oo ||   |         |o
Hyphozyma       GFDGRNETANQRG-TGGLLQLAEYIAGLSGGSWATASLTMNNWATTQSLKD---N---IW
Saccharomyces   AMDNRTDGANEHG-LGGLLQGATYLAGLSGGNWLTSTLAWNNWTSVQAIVDNTTESNSIW
Torulaspora     AMDNRTDGANEHG-LGGLLQSTTYLAGLSGGNWLVGTLAWNNWTSVQDIVNNMTEDDSIW
Penicillium     AFDSRTDNATATGHLGGLLQSATYISGLSGGSWLLGSIYINNFTTVDKLQTHEAG--SVW

                250       260       270       280       290       300
                  |         |         |         |         |         |
                ||  o                 |   o  o|  o|  |o|o  o |||
Hyphozyma       DLESNLIV-P-----EDG-KVSFYASILAAVAGKRNEGYQTSLTDYFGLSIADKILNGSM
Saccharomyces   DISHSILT-P--DGINIFKTGSRWDDISDDVQDKKDAGFNISLADVWGRALAYNFWPSLH
Torulaspora     DISNSIIN-P--GGFMIVTTIKRWDHISDAVEGKQDAGFNVSLTDIWGRALSYNFFPSLY
Penicillium     QFGNSIIEGPDAGGIQLLDSAGYYKDLADAVDGKKKAGFDTTLTDIWGRALSYQMFNASN

                310       320       330       340       350       360
                  |         |         |         |         |         |
              |     oo |      o     o o  |oo o oo  |o  o|o| oooo o o|  o|
Hyphozyma       YGNKFSVEWSDVKNTSKFTDASMPFPIIIADEREPGELIIPRNTTIWEFNPYEFGSWNPN
Saccharomyces   R-GGVGYTWSTLREADVFKNGEMPFPITVADGRYPGTTVINLNATLFEFNPFEMGSWDPT
Torulaspora     R-GGVAYTWSTLRDVEVFQNGEMPFPISVADGRYPGTQIIDLNATVFEFNPFEMGSWDPT
Penicillium     --GGLSYTWSSIADTPEFQDGDYPMPFVVADGRNPGELVIGSNSTVYEFNPWEFGTFDPT
```

FIG. 4b

```
                370        380        390        400        410        420
                 |          |          |          |          |          |
              |  o  |  oo|    |o        o| o||    |  oo||oo    o | |o     o
Hyphozyma     VSAFIPIEILGSSLDNGTSVLPDGVCVGGYETVAWVTGTSATLFSGLYLELISTSSNNII
Saccharomyces LNAFTDVKYLGTNVTNG-KPVNKGQCIAGFDNTGFITATSSTLFNQFLLRLNSTDLPSFI
Torulaspora   LNAFTDVKYLGTKVSNG-EPVNKGQCVAGYDNTGFIMGTSSSLFNQFLLQINSTSLPSFI
Penicillium   IFGFVPLEYLGSKFEGGSLPSNES-CIRGFDSAGFVIGTSSSLFNQFLLQINTTSLPSFI

                430        440        450        460        470        480
                 |          |          |          |          |          |
                  |      o||   oooo                    | |oooo o||o|oo
Hyphozyma     VDALKEIAQAVSNEQNDVSLV-PNPFYGYVG---EGDVQVSDLRNITLVDGGLDNENVPL
Saccharomyces ANLATDFLEDLSDNSDDIAIYAPNPFKEANFLQKNATSSIIESEYLFLVDGGEDNQNIPL
Torulaspora   KNLVTGFLDDLSEDEDDIAIYAPNPFKDTSYIQDNFSKSISESDYLYLVDGGEDNQNIPL
Penicillium   KDVFNGILFDLDKSQNDIASYDPNPFYKYN----EHSSPYAAQKLLDVVDGGEDGQNVPL

                490        500        510        520        530        540
                 |          |          |          |          |          |
              oo||  o |ooo o|o ooo    oo  ||o  o  o          |oo |oo  o
Hyphozyma     WPLVEPARDLDVIIAIDSSADVTN-WPNASALYQTSLRAQYPTYSQ--YAFPVMPDTNTV
Saccharomyces VPLLQKERELDVIFALDNSADTDDYWPDGASLVNTYQRQFGSQGLN--LSFPYVPDVNTF
Torulaspora   VPLVQDERNVDVIFALDNSADTDYYWPDGASLVSTYERQFSSQGLN--MSFPYVPDKRTF
Penicillium   HPLIQPERHVDVIFAVDSSADTDYFWPNGTSLVATYERSLNSSGIANGTAFPAVPDQNTF
```

FIG. 4c

```
                550       560       570       580       590       600
                 |         |         |         |         |         |
              |o oo  |o o|oo |  o o          o||oo|o    |    ooo||
Hyphozyma     VNRGLNTRPVFYGCNATVNVTNADTSFNGTKTPIIVYMPSYPYAAFADTSTFKL------
Saccharomyces VNLGLNKKPTFFGCDA-RNLTDLEYIP-----PLIVYIPNSRHSFNGNQSTFKMSYSDSE
Torulaspora   VNLGLADKPSFFGCDA-QNLTDLNYIP-----PLVVYIPNARHSYNSNTSTFKLSYTDDE
Penicillium   INLGLSTRPSFFGCDS-SNQTGPS--------PLVVYIPNAPYSYHSNISTFQLSTDDAE

                610       620       630       640       650       660
                 |         |         |         |         |         |

Hyphozyma     ------------------------------------------------------------
Saccharomyces RLGMIKNGFEAATMGNFTDDSDFLGCVGCAIIRRKQQNLNATLPSECSQCFTNYCWNGTI
Torulaspora   RLKMIKNGFEAATRGNLTDDSSFMGCVACAVMRRKQQSLNATLPEECSTCFTNYCWNGTI
Penicillium   RDNIILNGYEVATMANSTLDDNWTACVACAILSRSFERTGTTLPDICSQCFDRYCWNGTV

                670       680       690       700       710       720
                 |         |         |         |         |         |

Hyphozyma     ------------------------------------------------------------
Saccharomyces DSRSVSGVGNDDYSSSASLSASAAAASASASASASASASASASGSSTHKKNAGNALVNYSNL
Torulaspora   DDTPVSGLDNSDFDPTAASSAYSAYNTESYSSSSATGSKKNG-------AG------LPA
Penicillium   N------------STRPESYDPAFYLADNSMASVS----------------------L

                730
                 |

Hyphozyma     ------------------
Saccharomyces NTNTFIGVLSVISAVFGLI
Torulaspora   TPTSFTSILTLLTAIAGFL
Penicillium   PTMLSTVVAAGLAMLILV-
```

FIG. 4d

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0513709 A2	19/11/92	SE 0513709 T3	
		AT 120482 T	15/04/95
		CA 2068933 A,C	17/11/92
		CN 1066679 A	02/12/92
		DE 4115938 A	19/11/92
		DE 59201753 D	00/00/00
		ES 2072043 T	01/07/95
		HU 64578 A	28/01/94
		HU 213754 B	29/09/97
		PL 170548 B	31/12/96
		RU 2033422 C	20/04/95
		US 5264367 A	23/11/93
EP 0622446 A2	02/11/94	JP 7011283 A	13/01/95
		US 5532163 A	02/07/96
		CA 2122069 A	26/10/94